

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International BureauART CONSIDERED
NOT CITED

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|--|
| (51) International Patent Classification ⁶ : C12N 5/08, C07K 14/495, 14/51 | A1 | (11) International Publication Number: WO 98/35022 (43) International Publication Date: 13 August 1998 (13.08.98) |
| (21) International Application Number: PCT/US98/02137 (22) International Filing Date: 5 February 1998 (05.02.98) (30) Priority Data: 60/036,917 6 February 1997 (06.02.97) US (71) Applicant: OSIRIS THERAPEUTICS, INC. [US/US]; 2001 Aliceanna Street, Baltimore, MD 21231-2001 (US). (72) Inventor: CONNOLLY, Timothy, J.; 74 Pond Street, Belmont, MA 02178 (US). (74) Agents: HERRON, Charles, J. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US). | | (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> |
| (54) Title: p21 ^{CIP1} OR p27 ^{KIP1} EFFECTS ON THE REGULATION OF DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS (57) Abstract Disclosed is a method for distinguishing undifferentiated human mesenchymal stem cells (hMSCs) from partially or completely differentiated human mesenchymal cells. In accordance with the invention it has been discovered that the expression of p21 Cyclin Inhibitor Protein (p21 ^{CIP1}) is upregulated in partially or completely differentiated human mesenchymal cells as compared to undifferentiated hMSCs. Thus, this provides a quality control marker and test or assay to confirm that hMSCs are truly undifferentiated. That the p21 ^{CIP1} gene is either not expressed in clearly undifferentiated hMSCs or is significantly upregulated in partially or completely differentiated human mesenchymal cells by mesenchymal lineage inducers provides a screening method for identifying previously unknown mesenchymal lineage inducers. Also disclosed is an assay to determine the competence of mesenchymal progenitor cells to differentiate, particularly for <i>in vivo</i> tissue repair and particularly with respect to the osteogenic lineage. The inventors have made this possible by their observation that, in cells at approximately 80 % confluence in <i>in vitro</i> culture, p27 Kinase Inhibitor Protein (p27 ^{KIP1}) expression levels are upregulated in differentiation competent mesenchymal stem cell as compared to differentiation incompetent mesenchymal stem cells. | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|----|--------------------------|----|--|----|--|----|--------------------------|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | | | TR | Turkey |
| BG | Bulgaria | HU | Hungary | ML | Mali | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MN | Mongolia | UA | Ukraine |
| BR | Brazil | IL | Israel | MR | Mauritania | UG | Uganda |
| BY | Belarus | IS | Iceland | MW | Malawi | US | United States of America |
| CA | Canada | IT | Italy | MX | Mexico | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NE | Niger | VN | Viet Nam |
| CG | Congo | KE | Kenya | NL | Netherlands | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NO | Norway | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | NZ | New Zealand | | |
| CM | Cameroon | | | PL | Poland | | |
| CN | China | KR | Republic of Korea | PT | Portugal | | |
| CU | Cuba | KZ | Kazakhstan | RO | Romania | | |
| CZ | Czech Republic | LC | Saint Lucia | RU | Russian Federation | | |
| DE | Germany | LI | Liechtenstein | SD | Sudan | | |
| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

p21^{CIP1} OR p27^{KIP1} EFFECTS ON THE REGULATION OF DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

Background of the Invention

The osteogenic potential of bone marrow stromal cells has been demonstrated by various studies involving transplantation and culture systems *in vitro*. In particular, human mesenchymal stem cells are capable of differentiation into osteoblasts *in vivo*. Potential modulators of mesenchymal cellular differentiation have been identified. In particular, the synthetic glucocorticoid dexamethasone induces the expression of osteoblastic phenotypic markers in both immature osteoblasts and less committed mesenchymal progenitor cells (Maniopoulos *et al.*, 1988; Kasugui *et al.*, 1991; Saito, K. *et al.*, 1991; Haynesworth *et al.*, 1992). Currently, little is known about the molecular events which lead to the differentiation of mesenchymal cells into the osteoblastic lineage.

Growth factors of the transforming growth factor- β (TGF- β) superfamily have a wide range of activities in normal growth and development and have been strongly implicated in skeletal differentiation. Among this superfamily, the subfamily of bone morphogenic proteins (BMPs) plays an important role in bone formation. Several BMPs are thought to regulate the early commitment of mesenchymal cells to osteogenic and chondrogenic lineages due to the fact that BMPs are capable of inducing ectopic bone formation when injected into nonskeletal sites such as muscle (Urist, 1965; Wozney, 1992; Kingsley, 1994). Bone formation proceeds through a series of endochondral events which is initiated by the chemotaxis of mesenchymal

cells into the implantation site (Sampath and Reddi, 1981). The cells proliferate and differentiate into chondrocytes which are subsequently replaced by the deposition of bone. Thus, the BMP family of proteins perform important biochemical functions in a complex developmental process.

Since BMPs are secreted proteins, the characterization of their receptors and signal transduction pathways in marrow stromal cells is an important step toward understanding the role of these proteins in normal development. Recently, a family of receptors which bind BMPs has been identified in various species and shown to have conserved Ser/Thr kinase intracellular signaling domains. This family of receptor proteins can be further subdivided into either a type I or type II receptor based upon the degree of amino acid sequence similarity in the Ser/Thr kinase domain and extracellular cysteine residues. In particular, a serine/threonine kinase receptor from *Caenorhabditis elegans*, DAF 4, is a type II BMP receptor (Estevez *et al.*, 1993). DAF 4 is capable of binding both BMP 2 and BMP 4 (Estevez, 1993). In *Drosophila melanogaster*, saxophone (sax), thick veins (tkv) and punt have been cloned and shown to encode type I (*sax* and *tkv*) and a type II (*punt*) member of the TGF β family of receptors (Letsou *et al.*, 1995; Affolet *et al.*, 1994; Brummel *et al.*, 1994; Xie *et al.*, 1994). It is believed that these proteins are functional receptors for dpp, a BMP 2 homolog in *Drosophila*, as inactivation of either receptor is similar to a dpp null phenotype. Upon transient expression in Cos cells, tkv can bind dpp and punt can bind BMP 2 in the presence of either sax or tkv (Brummel *et al.*, 1994; Nellen *et al.*, 1994; Okano *et al.*, 1994). Recently, both a mouse (TFR-II) and a human (ALK 3) homolog of DAF 4 have been cloned by reverse transcription based polymerase chain reaction (RT-PCR) with degenerate oligonucleotides (ten Dijke *et al.*, 1993; Suzuki *et al.*, 1994; Koenig *et al.*, 1994). When expressed in Cos cells, the ALK 3 receptor is capable of binding both BMP 4 and BMP 7 but not activin or TGF β (ten Dijke *et al.*, 1994). In addition, the TFR-II gene when expressed in Cos cells is capable of binding both BMP 2 and BMP 4 but not TGF β or activin (Suzuki *et al.*, 1994). As a result, the ALK 3 and TFR-II receptors are postulated to be receptors for the BMP 2/4 ligands.

The p21^{CIP1} gene was first cloned and characterized as an important effector of p53 mediated cell cycle arrest (El-Diery, 1993; Xiong *et al.*, 1993; Harper *et al.*, 1993). The p21^{CIP1}

protein is capable of binding and inactivating Cdk-Cyclin complexes which exist in mammalian cells. Subsequently, the p21^{CIP1} protein was found to be upregulated during the differentiation of a number of mammalian cells both *in vitro* and *in vivo* (Jiang *et al.*, 1994; Steinman *et al.*, 1994; Halevy *et al.*, 1995; Macleod *et al.*, 1995; Parker *et al.*, 1995). In addition, the p21^{CIP1} has been shown to be induced by TGF β ligand and thus has been implicated as an effector of the TGF β signaling pathway. (Datto *et al.*, 1995). It has been suggested that the p21^{CIP1} protein is a principal mediator of the antiproliferative actions of a variety of differentiation signals and DNA checkpoint controls.

Several groups have demonstrated that p21^{CIP1} expression is upregulated during the *in vitro* differentiation of a variety of cell types (Jiang *et al.*, 1994; Steinman *et al.*, 1994; Halevy *et al.*, 1995; Macleod *et al.*, 1995). A variety of inducing agents, which include TPA, sodium butyrate, DMSO, and retinoids, have been shown to upregulate p21^{CIP1} expression in various cells.

Summary of the Invention

The present invention provides a method for distinguishing undifferentiated human mesenchymal stem cells (hMSCs) from partially or completely differentiated human mesenchymal cells. In accordance with the invention it has been discovered that the expression of p21 Cyclin Inhibitor Protein (p21^{CIP1}) is upregulated in partially or completely differentiated human mesenchymal cells as compared to undifferentiated hMSCs. Thus, this provides a quality control marker and test or assay to confirm that hMSCs are truly undifferentiated.

In one embodiment the mesenchymal cells are transfected with a construct comprising the DNA coding region for a detectable marker protein, such as firefly luciferase, under the control of a p21^{CIP1} promoter. The p21^{CIP1} promoter is activated to a greater extent in partially or completely differentiated human mesenchymal cells as compared to undifferentiated hMSCs, resulting in higher levels of marker expression. The level of marker expression is then observed. This aspect of the invention makes available an assay using hMSCs to evaluate the lineage induction potential of a variety of ligands.

In another embodiment the level of constitutive p21^{CIP1} protein that is being expressed by contacting the mesenchymal cells with anti-p21^{CIP1} protein antibody, monoclonal, polyclonal *etc.*, which antibody is directly or indirectly labeled and observing the amount of binding to p21^{CIP1} protein that has occurred.

In another aspect, the recognition by the inventors that the p21^{CIP1} gene is either not expressed in clearly undifferentiated hMSCs or is significantly upregulated in partially or completely differentiated human mesenchymal cells by mesenchymal lineage inducers provides a screening method for identifying previously unknown mesenchymal lineage inducers.

In another aspect, the invention provides an assay to determine the competence of mesenchymal progenitor cells to differentiate, particularly for *in vivo* tissue repair and particularly with respect to the osteogenic lineage. The inventors have made this possible by their observation that, in cells at approximately 80% confluence in *in vitro* culture, p27 Kinase Inhibitor Protein (p27^{KIP1}) expression levels are upregulated in differentiation competent mesenchymal stem cells as compared to differentiation incompetent mesenchymal stem cells.

In another aspect, the invention provides pharmaceuticals and methods for producing pharmaceuticals which induce or inhibit p21^{CIP1} or ALK 3. Compounds which induce elevated levels of p21^{CIP1} in cell systems where its level is reduced can be recognized as antineoplastic agents. In yet another aspect, the invention provides for agents which block ALK 3 receptor binding.

In yet another aspect, the invention provides for a rapid molecular *in vitro* assay to verify the quality control that hMSCs are indeed undifferentiated after isolation purification and/or culture expansion so that they may be shipped in commerce with regulatory (*e.g.*, FDA) clearance. In yet another aspect, the invention provides use of hMSC *in vivo* implants having a minimum amount of cell concentration of at least 3,000 adherent cells per square centimeter in the presence of added or locally administered *in vivo* osteogenic induction agents such as glucocorticoids.

Brief Description of the Drawings

Figure 1A-1B. Northern blot analysis of ALK 3 and the p21^{CIP1} expression upon dexamethasone treatment. In both Figures 1A and 1B the minus(-) sign indicated the absence of osteogenic supplement (dexamethasone) while the plus(+) sign indicates the presence of osteogenic supplement (dexamethasone) in the media.

Figure 1A: Human MSCs were cultured in either control media or media supplemented with 10⁻⁷ M dexamethasone for 48 hours. Total cellular RNA was extracted and 20 µg of RNA from either control cells or dexamethasone treated cells was subjected to electrophoresis through a formaldehyde gel, transferred to nylon membrane and then hybridized with radiolabelled probes for ALK 3 as indicated.

Figure 1B: Human MSCs were cultured in either control media or media supplemented with 10⁻⁷ M dexamethasone for 48 hours. Total cellular RNA was extracted and 20 µg of RNA from either control cells or dexamethasone treated cells was subjected to electrophoresis through a formaldehyde gel, transferred to nylon membrane and then hybridized with radiolabeled probes for p21^{CIP1} as indicated.

Figures 2A-2C. Immunohistochemical staining of human embryonic sections.

Figure 2A: A human embryonic section of a 76 day humerus was stained with a normal mouse IgG antibody.

Figure 2B: A human embryonic section of a 76 day humerus was stained with a monoclonal antibody against p21^{CIP1}.

Figure 2C: A human embryonic section of a 76 day humerus was stained with a monoclonal antibody against ALK 3.

Figures 3A-3B: BMP receptor activation of human MSC differentiation.

Figure 3A: Human MSCs were transfected with the p21^{CIP1} luciferase construct alone or together with the PCINeo control plasmid, PCINeo - ALK 3, PCINeo-ALK 3 plus the BMP-Type II receptor, PCINeo-ALK 3 DN plus the BMP Type II receptor construct or the BMP-Type II receptor, as indicated. Human MSCs expressing the luciferase gene from the p21^{CIP1} promoter were grown in DMEM/10% FBS for 2 days and then were lysed with a cell

culture lysis buffer (Promega). Luciferase activity in cell lysates was determined using a luciferase assay system (Promega) in conjunction with a Beckman liquid scintillation counter.

Figure 3B: A schematic representation of the 2.4 kb p21 promoter fragment fused to the luciferase reporter construct (WW-Luc), generously provided by Dr. Bert Vogelstein of Johns Hopkins University, is shown to indicate that the construct used in these studies contains a p53 binding site.

Figure 4: Protein levels of p21^{CIP1} in hMSCs and WI 38 cells. 100 µg of soluble protein were loaded per lane and lysates resolved on a 15% SDS-PAGE. Proteins were transferred to a PVDF membrane for Western blot analysis. The blot was probed with an antibody directed against the p21^{CIP1} protein (PharMingen) and visualized by the ECL system (Amersham) according to the manufacturer's instructions.

Figure 5: Human MSCs were transfected with GS-ALK 3 PCINeo or control EGFP PCINeo constructs in conjunction with p21-Luc and then cultured in DMEM/10% FBS for the period of times indicated. Firefly luciferase activity induced from the p21^{CIP1} promoter was measured using a Dual-Luciferase Reporter System (Promega).

Figures 6A-6B: Human MSCs were transfected with GS-ALK 3 PCINeo or control EGFP PCINeo constructs and then cultured in DMEM/10% FBS for the period of time indicated. Cells were harvested and alkaline phosphatase levels were quantitated by standard protocol.

Figure 6A: The results shown here are from MSCs that were not treated with dexamethasone.

Figure 6B: The results shown here are from MSCs that were treated with dexamethasone.

Figure 7: Protein levels of cell cycle regulatory proteins in hMSCs during culture. hMSCs were cultured under standard conditions for up to 8 days. Samples were harvested at

the indicated times and pelleted by centrifugation. Total protein was determined by Bradford analysis. Equivalent amounts of protein were resolved on a 12% SDS-PAGE, then transferred to a PVDF membrane for Western Blot analysis. The blot was probed with antibodies directed against the various cell cycle proteins as described and analyzed in conjunction with the ECL system (Amersham) according to the manufacturer's instructions.

Figure 8: Human MSCs were transfected with GS-ALK 3 PCINeo or control EGFP PCINeo constructs and then allowed to adhere to a culture dish upon overnight incubation. The cells were then trypsinized and plated at different densities corresponding to either a low (3000 cells cm²), a medium (9000 cells cm²) or a high (27,000 cells cm²) cell density. The cells were cultured in DMEM/10% FBS for 9 days, harvested and alkaline phosphatase levels were quantitated by standard protocol. The level of alkaline phosphatase activity in the graph is presented as the fold increase in alkaline phosphatase activity in GS-ALK 3 transfected MSCs as compared to MSCs transfected with the control construct.

Example 1

Human Mesenchymal Stem Cell Differentiation Screening

Materials and Methods

Human MSCs were cultured in either control or osteogenic supplemented medium for 2 days. Cells were grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with fetal bovine serum in a 37°C incubator with 5% CO₂. Osteogenic differentiation of the human MSCs was induced by supplementing the DMEM with (10⁻⁷ M) dexamethasone, 10mM β-glycerol phosphate and 50 ng/ml ascorbate. Total cellular RNA was isolated with guanidium isothiocyanate, phenol extraction and ethanol precipitation. Northern blot analysis was performed using 20 µg of total cellular RNA. The RNA was electrophoresed in a formaldehyde gel then transferred to a nylon membrane. The membrane was probed with random primed DNA probes that had been radiolabeled with [³²P dATP].

Human MSC λ ZAP cDNA libraries were constructed from RNA that had been isolated from either untreated human MSCs or MSCs that had been exposed to osteogenic supplemented medium for 48 hours.

The cDNA libraries were screened by degenerate PCR based methods to identify members of the Activin Like Kinase (ALK) receptors in human MSC populations. Polymerase chain reactions (PCR) were performed for 35 cycles consisting of 94°C for 1 minute, 54°C for 1 minute and 72°C for 3 minutes using degenerate primers that corresponded to structurally conserved subdomains VIII and XI in this protein family. Human MSC cDNA as a template and 5' GATCGAATTTCG(ACGT)TA(CT)(ACT)T(ACGT)GC(ACGT)CC(ACGT)GA 3' and 5' ATGGATCC(GA)T(CT)(ACGT)TG(AG)T(CA)CCA(AG)CA(TC)TC 3' oligonucleotide primers were used in the reaction. PCR products were cloned into Bluescript (KS) plasmids (Stratagene) and sequenced using Sequenase (U.S. Biochemical).

Transient transfections of human MSCs were performed by standard electroporation methods. Briefly, human MSCs were grown in monolayer culture in 10% FBS/DMEM media until 80% confluent, harvested by trypsinization and then resuspended in medium consisting of 10% FBS/DMEM high glucose, 20 mM HEPES at a concentration of 2.5×10^6 /ml. The cells were preincubated in this medium for 30 minutes. Electroporation was performed by mixing 250 μ l of the cell suspension with 20 μ g of plasmid DNA, 40 μ g of salmon sperm DNA as carrier and subjecting the 0.4 cm cuvette to 200 V constant voltage. Transfected human MSCs were grown in DMEM/10%FBS for 3 days and then were lysed in cell culture lysis buffer (Promega). Luciferase activity in cell lysates was determined using a luciferase assay system (Promega) in conjunction with a Beckman liquid scintillation counter.

Immunohistological analysis of human embryonic sections was performed using a monoclonal antibody generated against the human p21^{CIP1} protein (Pharmingen) and a polyclonal antibody that was generated against the ALK 3 receptor. Briefly, the tissue sections were treated with pepsin (0.5 mg/ml) for 30 minutes at room temperature and then blocked with a goat polyclonal antiserum for 1 hour. Primary antibody was diluted in TBS/1%BSA and incubated with the tissue section for 1 hour. Tissue sections were washed in TBS and then probed with a biotin conjugated secondary antibody for 1 hour at room temperature. Tissue specific expression of the proteins was visualized by the addition of streptavidin-peroxidase and diaminobenzadine.

Results

In order to identify members of the TGF β family of serine/threonine kinase receptors, we employed a polymerase chain reaction as described above. A PCR reaction was run on cDNA which had been isolated from cells that had been grown in osteogenic supplemented media using degenerate primers. This amplified a predicted 300 bp fragment (cDNA from untreated cells gave no PCR product). The PCR product was subcloned and then subjected to sequence analysis. The sequence analysis revealed that the PCR fragment corresponded to a partial cDNA encoding the ALK 3 receptor. In each case, the PCR reactions failed to amplify an ALK 3 fragment using cDNA derived from untreated human MSCs as a template. These results suggest that the ALK 3 receptor is present in human MSCs that had received osteogenic

supplemented media.

We investigated the expression of the ALK 3 receptor in the human MSC populations. For this purpose, approximately 1×10^6 plaques from each of an undifferentiated and a dexamethasone induced human MSC cDNA library were plated on NZYM plates, transferred to nitrocellulose membranes and then subjected to a low stringency hybridization analysis using the ALK 3 PCR fragment as a probe. Through this low stringency screening approach, we identified 5 positive clones from OS treated human MSCs. No ALK 3 positive clones were detected by low stringency screening of the CDNA library that corresponded to undifferentiated human MSCs. The results from such a hybridization analysis suggest that the ALK 3 gene is likely not expressed in undifferentiated human MSCs or its expression is dramatically upregulated upon dexamethasone treatment.

Example 2

Northern Analysis of p21 mRNA

In order to further confirm this observation, the expression pattern of the ALK 3 receptor mRNA transcript in human MSCs undergoing *in vitro* osteogenic differentiation was examined by direct Northern analysis. Northern blot analysis was performed on total cellular RNA that had been extracted from human mesenchymal stem cells that received either no osteogenic supplement or osteogenic supplement (dexamethasone) for 48 hours (Figures 1A and 1B). Total RNA was subjected to electrophoresis on a 1% agarose/formaldehyde gel, transferred to a nylon membrane and was then probed with a fragment corresponding to the ALK 3 cDNA (Figure 1A) or the p21^{CIP1} cDNA (Figure 1B). The results indicate that the message for the ALK 3 receptor was detectable in RNA that had been extracted from MSCs that had received osteogenic supplement. In addition, the results indicate that the p21^{CIP1} transcript is present in both undifferentiated MSCs and MSCs that had received osteogenic supplement. The results also indicate that the p21^{CIP1} and the ALK 3 transcripts are increased in RNA that was isolated from MSCs that were exposed to dexamethasone (Figure 1A and Figure 1B).

Example 3

Expression Patterns of p21^{CIP1} and ALK 3 Proteins

We next investigated the expression patterns of the ALK 3 protein and p21^{CIP1} protein in human MSCs undergoing osteogenic differentiation *in vivo*. For this purpose, tissue from 76 day human embryonic sections was obtained and probed with control antibodies (Figure 2A) and antibodies generated against the p21^{CIP1} and ALK 3 proteins (Figures 2B and 2C). Histologic sections from a 76 day embryonic humerus were probed with either a control antibody or a monoclonal antibody generated against the p21^{CIP1} protein (Pharmingen). The results demonstrate that the p21^{CIP1} protein was clearly expressed in the more differentiated region of the developing periosteum (Figure 2B). In addition, the p21^{CIP1} protein was expressed in fully developed osteocytes that had been encased in an osteoid matrix (Figure 2B). The less differentiated region of the periosteum where undifferentiated MSCs are detected, no p21^{CIP1} protein was detected (Figure 2B). Figure 2B also shows undifferentiated periosteum and hypertrophic cartilage.

The results demonstrate that both the p21^{CIP1} protein and the ALK 3 receptor are expressed in the developing periosteum. To determine if a functional relationship existed between p21^{CIP1} and ALK 3 expression in human MSCs undergoing osteogenic differentiation, we explored an *in vitro* transcription assay. For this purpose, we constructed an expression vector which expressed either the full length (pCINeo-ALK 3) or a signaling defective form (pCINeo-ALK 3 DN) of the human ALK 3 receptor cDNA in human MSCs under the control of the cytomegalovirus (CMV) promoter. A series of transient transfections were performed with human MSCs with a plasmid containing the p21^{CIP1} promoter fused in front of a luciferase reporter gene.

When either the vector, the ALK 3 or the BMP type II receptor alone was transfected into human MSCs, background luciferase levels were obtained (Figure 3). Upon transient transfection of the ALK 3 receptor in conjunction with the type II BMP receptor in human MSCs, a significant increase in luciferase activity was observed (Figure 3A). In contrast, transient transfection of the signaling defective form of the receptor together with the type II BMP receptor reduced the luciferase activity to background levels (Figure 3A). These results

demonstrate that a functional relationship between ALK 3 expression and p21^{CIP1} expression exists in human MSCs. In addition, the luciferase assays indicate that undifferentiated MSCs do not express functional (or functional amounts of) type I and type II receptors as ectopic expression of either BMP receptor alone does not induce p21^{CIP1} expression. In contrast, ectopic expression of a BMP type I together with a BMP type II receptor induces a luciferase response through the p21^{CIP1} promoter in human MSCs.

Figure 3A shows BMP receptor activation of human MSC differentiation. Human MSCs were transfected with the p21^{CIP1} luciferase construct alone or together with the PCINeo control plasmid, PCINeo - ALK 3, PCINeo-ALK 3 plus the BMP type II receptor, PCINeo-ALK 3 DN plus the BMP type II receptor construct or the BMP type II receptor as indicated. Human MSCs expressing the luciferase gene from the p21^{CIP1} promoter were grown in DMEM/10% FBS for 2 days and then were lysed with a cell culture lysis buffer (Promega). Luciferase activity in cell lysates was determined using a luciferase assay system (Promega) in conjunction with a Beckman liquid scintillation counter.

Figure 3B shows a schematic representation of the 2.4 kb p21^{CIP1} promoter fragment fused to the luciferase reporter construct. The construct used in these studies contains a p53 binding site as indicated.

Example 4

Human MSCs have Reduced Levels of p21^{CIP1} Protein Compared to a Human WI 38 Fibroblast Cell Line

We have performed a preliminary Western blot analysis of p21^{CIP1} protein levels in human MSCs and human WI 38 fibroblasts. Total cell lysates were prepared from culture expanded human MSCs and protein concentration was determined by standard Bradford analysis. 100 µg of soluble protein from the human MSC and WI 38 cell lysates were resolved on SDS PAGE, then transferred to a PVDF membrane for Western blot analysis. The membranes were probed with a monoclonal antibody (Pharmingen) that is directed against the p21^{CIP1} protein. The results indicate that the p21^{CIP1} protein is clearly detectable in 100 µg of cellular lysate prepared from a WI 38 fibroblast. The p21^{CIP1} protein is substantially reduced in

100 µg of cellular lysate from human MSCs when compared to the level in a human WI 38 cell line.

We quantitated the intensity of the p21^{CIP1} bands by densitometric analysis and found that the WI 38 fibroblast had an approximately 4 fold increase in p21^{CIP1} protein levels compared to human MSCs.

A large body of evidence indicates that an increase in p21^{CIP1} protein levels is a marker of cellular differentiation *in vivo*. This data when taken together suggests that human MSCs may have substantially lower levels of the p21^{CIP1} protein than a differentiated mesenchymal cell.

Example 5

hMSCs Respond to BMP Receptor Signaling

We investigated the ability of the GS-ALK 3 to stimulate p21^{CIP1} promoter activity. Human MSCs were transfected with the GS-ALK 3 or vector control in conjunction with a p21^{CIP1}-luciferase promoter construct (WW-Luc) and then cultured in DMEM/10% FBS for a period of 7 days. At this timepoint, the cells were harvested and assayed for luciferase activity as a marker of cellular differentiation. The results demonstrate that the undifferentiated MSC is capable of responding to BMP receptor signaling by stimulating p21^{CIP1} promoter activity. See Figure 5.

Example 6

Human MSCs are Responsive to BMP Receptor Signaling in the Absence of Osteogenic Supplemented Media.

Recent results suggest that the undifferentiated MSCs are capable of responding to BMP receptor signaling. The ALK 3 type Ia BMP receptor was cloned and then was used as a reagent to investigate the effects of BMP/BMP receptor signaling on human MSCs. Due to the unavailability of a BMP ligand, we mutated the ALK 3 receptor by site directed mutagenesis and rendered it constitutively active (GS-ALK 3). As a consequence of this mutation, the GS-ALK 3 construct has been shown to transduce an intracellular signal in the absence of binding

BMP ligand in other mammalian cells. Human MSCs were transfected with the GS ALK 3 or control constructs and then cultured in either DMEM/10% FBS or osteogenic supplemented media for a period of 12 days. At various time points, namely 4, 8 and 12 days, the cells were harvested and assayed for alkaline phosphatase activity as a marker of osteogenic differentiation.

The results presented here demonstrate that the undifferentiated human MSCs after 4 days of logarithmic growth in cell culture are initially unresponsive to BMP receptor signaling as indicated by a failure to upregulate alkaline phosphatase levels at day 4 of culture. The MSCs however are capable of responding to BMP receptor signaling after 8 and 12 days of culture by stimulating alkaline phosphatase levels. The GS-ALK 3 receptor kinase is capable of upregulating alkaline phosphatase levels in cells grown in the absence or presence of osteogenic supplemented media. See Figures 6A and 6B.

We attempted to investigate the nature of this BMP receptor responsiveness in MSCs through examination of the cell cycle machinery in MSCs. We cultured cells in DMEM/10% FBS and isolated cells at different time points in the process as indicated. Cell lysates were made from the cells and the proteins were resolved by SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was probed with antibodies directed against various cell cycle proteins. A Western blot analysis of the cell cycle machinery demonstrated that the p27 levels increase in response to logarithmic growth in culture. See Figure 7. Thus, there is a correlation between an increase in p27 levels and the ability of human MSCs to respond to BMP receptors as indicated by an upregulation of alkaline phosphatase levels.

We also investigated the nature of this BMP receptor responsiveness by culturing MSCs at different densities. Human MSCs were transfected with either the GS-ALK 3 or a control construct and then cultured in DMEM/10% FBS at different cell densities corresponding to either a low, a medium or a high cell density. After 10 days in culture the cells were harvested and assayed for alkaline phosphatase activity as a marker of osteogenic differentiation. The results indicate that the GS-ALK 3 transfected MSCs when cultured at a high cell density are capable so stimulating phosphatase activity 16 fold over MSCs receiving a control construct. In contrast, the MSCs transfected with the GS-ALK 3 construct and cultured at a low cell density,

stimulated alkaline phosphatase activity only 2 fold over MSCs receiving a control construct. The results indicate that cellular density is an important criterion that controls the ability of undifferentiated MSCs to respond to BMP receptor signaling.

Discussion

This study demonstrates that undifferentiated MSCs do not express functional (or functional amounts of) type I and type II BMP receptors. In addition, the results demonstrate that human MSCs transcriptionally upregulate a type I BMP receptor, ALK 3 in response to dexamethasone treatment. Furthermore, the transcriptional upregulation of the ALK 3 receptor is correlated with an upregulation of p21^{CIP1} expression in human MSCs undergoing both *in vitro* and *in vivo* differentiation.

In particular, the results described above indicate that an undifferentiated human MSC population is unresponsive to exogenous BMP factors due to the fact that undifferentiated human MSCs do not express functional type I and type II BMP receptors. However, ectopic expression of the ALK 3 receptor, thus mimicking transcriptional ALK 3 upregulation, in conjunction with ectopic expression of a type II BMP receptor stimulates p21^{CIP1} transcription. It appears that the p21^{CIP1} Cdk inhibitor is a central target of the diverse set of inducers of cellular differentiation. It is possible that the p21^{CIP1} promoter contains multiple cis acting elements that are responsive to a variety of differentiation signals.

The transient transfection studies indicate that the ALK 3 receptor in conjunction with a type II BMP receptor is capable of transducing an intracellular signal to induce the differentiation of human MSCs. Thus, it appears that the requisite molecular machinery for BMP receptor signaling exists in an undifferentiated human MSC population. When taken together, these results strongly suggest that osteogenic differentiation of human MSCs, both *in vitro* and *in vivo*, is controlled in part by particular BMPs.

This demonstration that co-expression of a type I and type II BMP receptor in an undifferentiated human MSC stimulates p21^{CIP1} transcription also provides an assay to investigate the effects of various BMPs on human MSC differentiation. The assay can be used

to identify particular BMP ligands which signal human MSC differentiation. In addition, the assay can be used to investigate the intracellular signaling events that are induced by either ectopic BMP receptor expression or the addition of BMP to responsive mesenchymal stem cell populations. In this regard, it will be interesting to perform both a mutation and deletion analysis of the human p21^{CIP1} promoter to define sequences required for both the dexamethasone and the ALK 3 induced increase in p21^{CIP1} expression. Both dexamethasone and the BMP receptor may induce *in vitro* osteogenic differentiation through direct upregulation of a transcription factor which induces p21^{CIP1} expression in human MSCs. Alternatively, dexamethasone may act through direct upregulation of the ALK 3 receptor and a type II BMP receptor which would, in turn, induce p21^{CIP1} expression in human MSCs. The transcriptional upregulation of these receptors would enable the cell to respond to endogenous BMP ligands in the culture media, include p21^{CIP1} transcription and subsequent osteogenesis.

Furthermore, the described assay has utility as a high throughput screen to identify agents that would induce the undifferentiated MSCs to express BMP receptors. Once BMP receptors are expressed, the evidence presented indicated that the hMSC becomes responsive to endogenous BMP ligands in the surrounding environment. We have demonstrated that one synthetic glucocorticoid, dexamethasone, upregulates ALK 3 expression in human mesenchymal stem cells. The identification of additional factors which would upregulate BMP receptors in human MSCs would therefore be valuable.

The results presented indicate that the undifferentiated MSC cell has lower levels of p21^{CIP1} protein than a more differentiated mesenchymal derivative. In response to MSC cellular differentiation there is an upregulation of p21^{CIP1} transcription. In addition, we observe that there is substantially less p21^{CIP1} protein in a human MSC than in a human WI 38 fibroblast. In this regard, the measurement of p21^{CIP1} protein and p21^{CIP1} promoter activity may provide an assay to evaluate an undifferentiated MSC population.

Cited Literature

Brummel, T.J., Twombly, V., Marques, G., Wrana, J.L., Newfeld, L., Massague, J., Oconner, M.B. and Gelbart, W.M. 1994 Characterization and relationship of dpp receptors encoded by the saxophone and thick vein genes in *Drosophila*. *Cell* 78, 251-261.

Datto, M., Yu, Y., and Wang, X.F. 1995. Functional analysis of the transforming growth factor b responsive elements in the WAF1/Cip1/p21 promoter. *J. Biol. Chem.* 270, 28623-28628.

Estevez, M.L., Attisano, L., Wrana, J., Albert, P.S., Massague, J., and Riddle, D.L. 1993. The *Daf-4* gene encodes a bone morphogenic protein receptor controlling *C. elegans* dauer larva development. *Nature* 365, 644-649.

El Diery, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., J-rent J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817-825.

Halevy, O., Novitch, B., Spicer, D., Sapek, S., Rhee, J., Hannaon, G., Beach, D., and Lassar, A. (1995). Correlation of Terminal Cell Cycle Arrest of Skeletal Muscle with Induction of p21 by MYOD. *Science* 267 1018-1021.

Harper, J.W. Adami, g.R., Wei, N., Keyomarsi, K. And Elledge, S.J. (1993). The p21 Cdk interacting protein Cip1 is a potent inhibitor of G1 cyclin dependent kinases. *Cell* 75, 805-816.

Haynesworth, S.E., Goshima, J., Goldberg, V., and Caplan, A. (1992). Characterization of cells with osteogenic potential from human marrow. *Bone* 13, 81-88.

Jiang, H., Lin, J., Zao-zhong, S., F., Collart, Huberman, E. And Fisher, P. (I 994). Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21 WAF I /CIP I expression in the absence of p5 3. *Oncogene*

Kasugi, S., Todescan, R., Nagata, T., Yao, K., Butler, W.T., and Sodek, J. (1991). Expression of bone matrix proteins associated with mineralized tissue lortnation by adult rat bone marrow cells in vitro: Inductive effects of dexamethasone on osteoblastic phenotype. *J. Cell Physiol.* 147, 111-120.

Koenig, B.B., Cook, J.S., Wolsing, D.H., Ting, J., Teisman, J.P., Correa, P.E., Olson, C.A., Pecquet, A.L., Ventura, F., Grant, R.A., Chen, G-X., Wrana, J., Massague, J., and Rosenbaum, J. S. (I 994). Characterization and cloning of a receptor for BMP 2 and BMP 4 from NIH 3T3 cells. *Mol. Cell. Biol.* 14, 5961-5974.

MacLeod, K.E, Sherry, N., Harmon, g., Beach, D., Tokino, T., Kinzler, K., Vogelstein, b., and Jacks, T. 1995. P53 dependent and independent expression of p21 during cell growth, differentiation and DNA damage. (1995) *Genes and Dev.* 9, 935-944.

- Maniopoulos, C., Sodek, J., and Melcher, A.H. (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res.* 254 317330.
- Parker, S.B., Eichele, G., Zhang, P., Rawls, A., Sands, A.T., Bradley, A., Olson, E.N., Harper, J.W., and Elledge, S.J. (1995). p53 independent expression of p21CIP1 in muscle and other terminally differentiated cells. *Science.* 267, 1024-1027.
- Satsumura, K., and Nagayama, M. (1991). Ultrastructure of mineralized nodules formed from rat bone marrow stromal cell culture in vitro. *Acta Anat.* 142, 97-104.
- Steinman, R., Hoffman, B., Iro, A., Guillof, C., Leiberman, D., and El-Housein, M. (1994) Induction of p21 (WAF1/CIP1) during differentiation. *Oncogene* 9.3389-3396.
- Xiong, Y., Hannon, G., Zhang, H., Casso, D., Koybayashi, R., and Beach, D. (1993). P21 is a universal inhibitor of cyclin kinases. *Nature* 366. 701-704.
- Urist, M.R. (1965). Bone Formation by autoinduction. *Science* 150, 893-899.
- Sampath, T.K., Reddi, H. (1991). Dissociative extraction and reconstitution of extracellular matrix components involved in local bone formation. *Proc. Nat. Acad. Sci.* 78, 7599-7603.
- Suzuki, A., Thies, R.S., Yamaji, N., Song, J., Wozney, J., Murakami, K., and Ueno, N (1994). A truncated bone morphogenic protein receptor affects dorsal ventral patterning in the xenopus embryo. *Proc. Nat. Acad. Sci.* 91, 10255-10259.
- ten Dijke, P.H., Ichijo, H., Franzen, P., Schulz, P., Sara, H., Toyoshima, H., Heldin, C.H., and Miyazono, K. (1993). Activin receptor like kinases: A novel subclass of cell surface receptors with predicted serine/threonine kinase activity. *Oncogene* 8, 2879-2887.
- ten Dijke, P.H., Yamashita, T.K., Sampath, T.K., Reddi, A.H., Estevez, M., Riddle, D.L., Ichijo, H., Heldin, C.H. and Miyazono, K. (1994). Identification of type I receptors for osteogenic protein 1 and bone morphogenic protein 4. *J. Biol. Chem.* 269, 16985-16988.
- Wozney, J.M. (1992). The bone morphogenic protein family and osteogenesis. *Mol. Reprod. Dev.* 32, 160-167.
- Xie, T., Finelli, A.L., Padgett, R.W. 1994. The drosophila saxophone gene: A serine/threonine kinase receptor of the TGF- β superfamily. *Science* 263 1756-1759.

CLAIMS

1. A method for distinguishing undifferentiated human mesenchymal stem cells from partially or completely differentiated human mesenchymal cells comprising measuring the upregulation of p21^{CIP1} expression in partially or completely differentiated human mesenchymal cells.
2. The method of claim 1 wherein the human mesenchymal stem cells are modified with a genetic construct comprising the DNA coding region for a detectable marker protein and a p21^{CIP1} promoter.
3. The method of claim 2 wherein the level of marker expression measured is correlatable to p21^{CIP1} activation.
4. The method of claim 1, wherein the level of constitutive p21^{CIP1} protein expression is determined by contacting the cells with labelled anti-p21^{CIP1} protein antibody.
5. A method for identifying a mesenchymal stem cell lineage inducer comprising contacting an undifferentiated mesenchymal stem cell line with a factor suspected of being a mesenchymal stem cell lineage inducer; and measuring the upregulation of p21^{CIP1} expression in human mesenchymal cells, if any.
6. The method of claim 5 wherein said factor is a growth factor.
7. The method of claim 6 wherein said factor is a bone morphogenic protein.
8. A method for determining the ability of mesenchymal progenitor cells to differentiate comprising measuring the upregulation of p27^{KIP1} expression in the mesenchymal cells.
9. A method for identifying an agent that induces undifferentiated human mesenchymal stem cells to express bone morphogenic protein receptors.

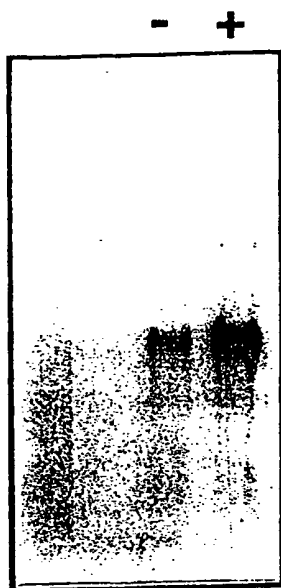


Figure 1

2/8



Figure 2A.



Figure 2B



Figure 2C

4/8

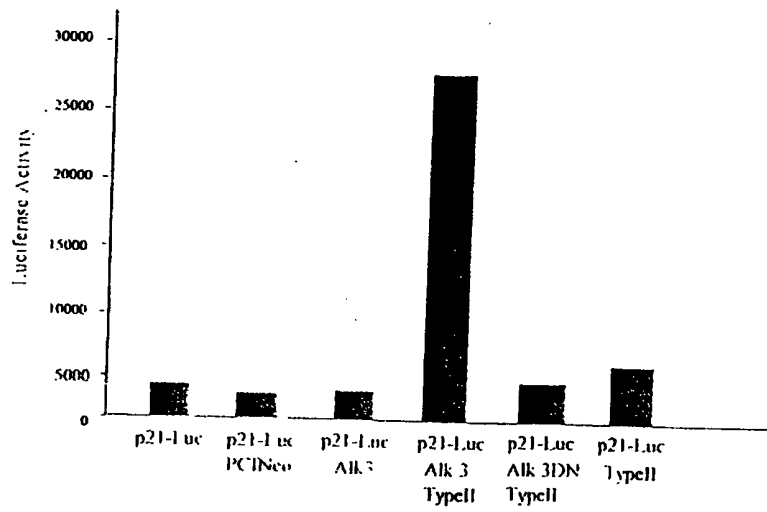


Figure 3A



Figure 3B

p21^{waf1} levels in hMSCs and WI38 cells

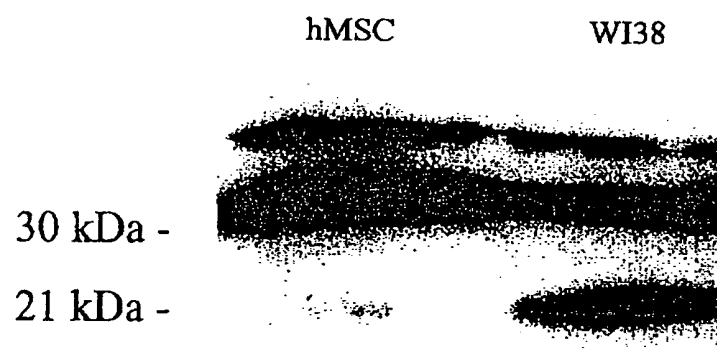
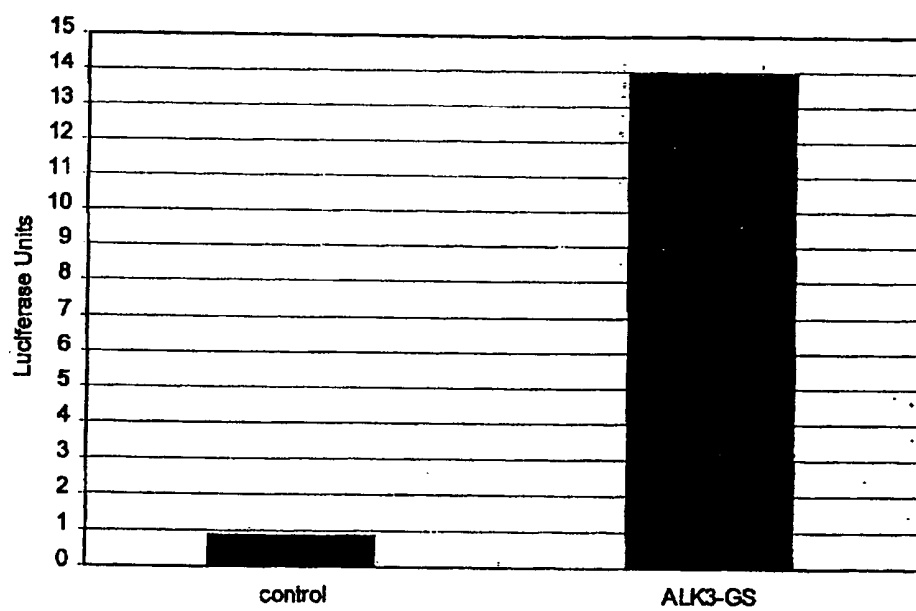


Figure 4

**Figure 5**

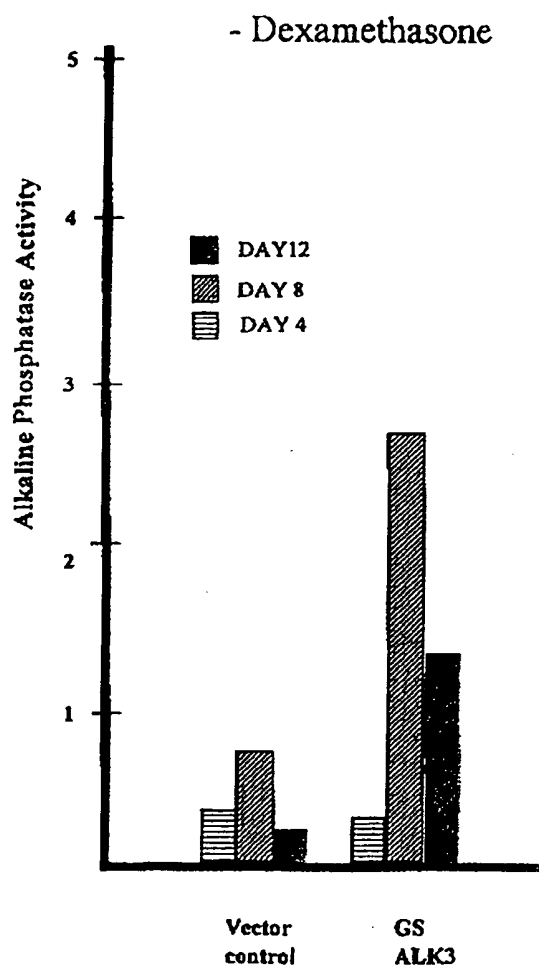


Figure 6A

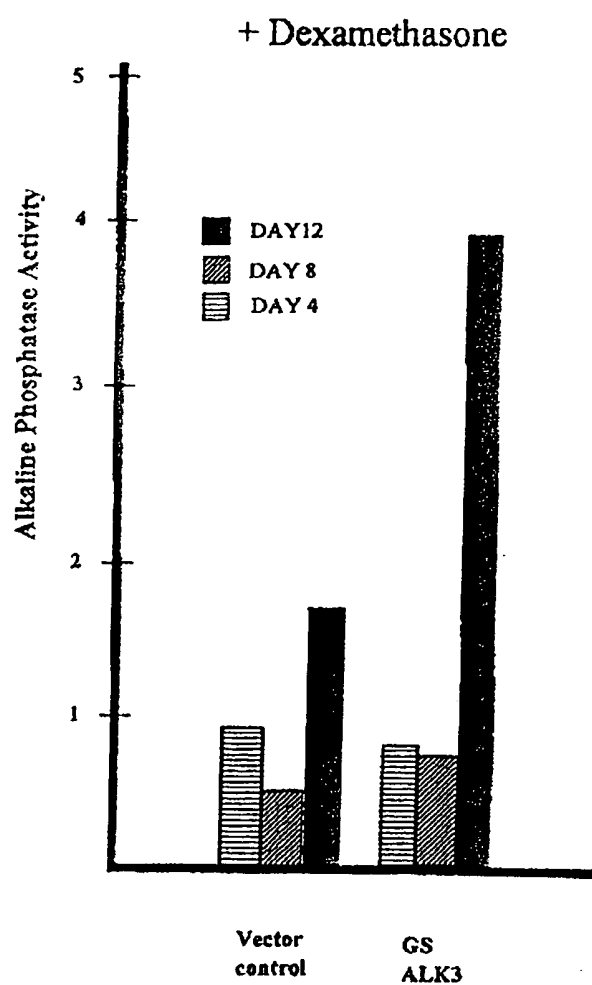


Figure 6B

8/8

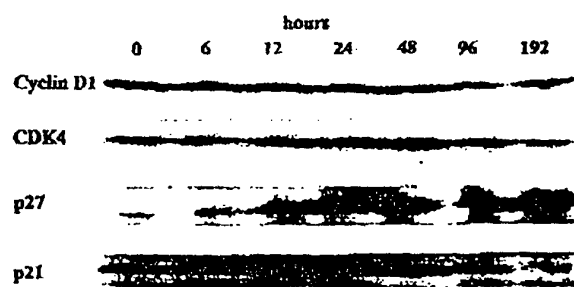


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/02137

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/08; C07K 14/495, 14/51

US CL :530/350, 21; 435/372, 377

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 21; 435/372, 377

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| Y | US 5,486,359 A (CAPLAN et al) 23 January 1996, see entire document. | 1-9 |
| Y | AHRENS, M. et al. Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T 1/2 Cells induces differentiation into distinct mesenchymal cell lineages. DNA and Cell Biology. 1993, Vol. 12, No. 10, pages 871-880, see entire document. | 1-9 |
| Y | JOYCE, M. E. et al. Transforming growth factor - β and the initiation of chondrogenesis and osteogenesis in the rat femur. J. Cell Biol. June 1990, Vol. 110, pages 2195-2207, see entire document. | 1-9 |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

| | | |
|---|-----|--|
| * Special categories of cited documents: | *T | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *X | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *B* earlier document published on or after the international filing date | *Y | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *A* | document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | | |
| *P* document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

30 MARCH 1998

Date of mailing of the international search report

27 MAY 1998

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARY TUNG

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/02137

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| Y | SAVATIER, P. et al. Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. <i>Oncogene</i> . 1995, Vol. 12, pages 310-322, see entire document. | 1-9 |
| Y | TIKOO, R. et al. Changes in cyclin-dependent kinase 2 and p27 ^{KIP1} accompany glial cell differentiation of central glia-4 cells. <i>J. Biol. Chem.</i> 03 January 1997, Vol. 272, No. 1, pages 442-447, see entire document. | 1-9 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/02137

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, EMBASE, PATOSWO, SCISEARCH, APS, JPO, EPO

Mesenchyma, stem cells, pluripotent cells, progenitor cells, p21CIP1, cyclin inhibiting protein, p27KIP1, kinase inhibiting protein.